Journal of Bioresource Management

Volume 1 | Issue 2 Article 1

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Anam Rasool
Fatima Jinnah Women's University, Rawalpindi, anam.rasool32@gmail.com

Shazia Irum
Fatima Jinnah Women's University, Rawalpindi

Recommended Citation
Rasool, A., & Irum, S. (2014). Toxic Metal Effect on Filamentous Fungi Isolated from the Contaminated Soil of Multan and Gujranwala, *Journal of Bioresource Management, 1* (2).
DOI: 10.35691/JBM.4102.0006
ISSN: 2309-3854 online

Toxic Metal Effect on Filamentous Fungi Isolated from the Contaminated Soil of Multan and Gujranwala

J. Bioresource Manage. (2014) 1(2): 38-51.

TOXIC METAL EFFECT ON FILAMENTOUS FUNGI ISOLATED FROM THE CONTAMINATED SOIL OF MULTAN AND GUJRANWALA

Anam Rasool* and Shazia Irum

Department of Environmental Sciences, Fatima Jinnah Women University, Rawalpindi, Pakistan.

***Email:** anam.rasool32@gmail.com.

ABSTRACT

Considering the importance of filamentous fungi for bioremediation of wastewater and contaminated soils, this study was planned to investigate the metal tolerance potential of indigenous filamentous fungi. Certain metals are important to biological actions. However all metals, whether essential or inessential will show toxicity at certain levels. During 2012 total 17 fungi were isolated and preserved from contaminated peri-urban agricultural areas of Multan and Gujranwala for further detail investigation of heavy metal tolerance. *Aspergillus niger, Aspergillus fumigatus and Aspergillus flavus* isolated from both soil and water samples while *Aspergillus terreus* and *Penicillium sp* were only isolated from soil samples of Multan and *Aspergillus versicolor, Aspergillus flavus, Fusarium oxysporum, Aspergillus niger* which were isolated from contaminated soils and water samples while *Penicillium sp* was isolated from only water samples of Gujranwala. These few fungal isolates were selected for tolerance to metal Cu $(SO_4)_2.5H_2O$, Cd $(NO_3)_2$, Cr $(NO_3)_2$ and Pd $(NO_3)_2$. The tolerant strains were selected with increasing metals concentration of 100ppm and compared to control in the medium. The degree of tolerance was measured by radial growth (cm) in the presence of various heavy metals and compare to the control, which contain no heavy metals. The present study investigation concludes isolates *Penicillium sp* and *Aspergillus flavus* isolated from soil of Gujranwala *show* maximum tolerance index 2.1 at 100ppm toward Cr and 4.8 at 100ppm toward Cd respectively. *Aspergillus Versicolor* (isolated from waste water) exhibit considerable highest tolerance index

J. Bioresource Manage. (2014) 1(2): 38-51.

toward Cu and Pb while show a sensitivity against other metals. From all the collected samples the Gujranwala soil and water show more tolerance toward the heavy metals as compared to Multan area.The present study indicates that in future similar strains will be tested with other heavy metals for the confirmation of tolerance and tolerant strains will be used for bioremediation of heavy metal.

Keywords: Filamentous fungi, bioremediation, metal tolerance, soil and water fungi, tolerance index.

INTRODUCTION

Heavy metals are environmental contaminants and not a new phenomenon. Heavy metals contamination is a major problem of our environment and they are also one of the major contaminating agents of our food supply (Gholizadeh et al., 2009; Khair, 2009). Heavy metals are an imprecise term used to describe more than dozen elements that are metals or metalloids (elements that have both metals and non metal characteristics). Example of heavy metals includes cadmium, lead, mercury copper, nickel, and manganese. Generally, heavy metals have densities above 5 g/cm³ (Adriano *et al*. 2005). They are an essential part of all living organisms and also present naturally in trace amount in our soil. The man made sources of metal contamination are mainly associated with certain industrial activities, agricultural practices, automobile emissions, coal fired power generation plants, municipal incinerators (Rattan *et al*., 2002; Marshall *et al*., 2003).

Toxic metals are metals that are poisonous soluble compounds and they are not essential minerals and have no other (?) biological role. Throughout the world, a major environmental problem is a heavy metals contamination, but due to their technical importance they are used in many industries and waste water from these industries has perpetual toxic effects on human beings and the environment (Anon., 2004) because they are constant in all parts of the environment and cannot be tarnished or destroyed easily. Toxic heavy metals arriving in the ecosystem may cause the geo-accumulation, bioaccumulation and biomagnifications. Heavy metals like Fe,

J. Bioresource Manage. (2014) 1(2): 38-51.

Cu, Zn, Ni and other trace elements are necessary to perform the proper functions of all biological systems but their deficiency or excess amount could lead to a number of disorders (Ward, 1995). In many areas of Pakistan, especially in big cities, industrial units are established without Environmental Impact Assessment and Planning (EIAP). The air, soil and water are increasingly polluted by industrial pollutants such as organic and inorganic chemicals and toxic metals (Irshad *et al.*, 1997). Heavy metals contaminated land is increasingly becoming an environmental, health, economic and planning issue in Pakistan (Hussain *et al.*, 1996).

Soil is a thin layer of material on the Earth's surface in which plants have their roots. Soil is a major group of microorganisms and main habitat for all species. The soil micro biota is involved in the breakdown and production of organic compounds as well as it dynamically involved in the cycling of plant nutrients and in the weathering of primary minerals (Parkinson & Coleman, 1991).

Fungus is one of the members of group of eukaryotic organisms and is classified as kingdom. Fungus includes yeasts, molds and mushrooms and is separated from bacteria, plants and animals. There are many differences between plants and fungi. One of the important differences is that fungal cell walls contain chitin and plants cell wall contains cellulose (Bowman *et al.*, 2006). Due to this major difference and some other differences fungi are grouped separately in a separate kingdom and are named as *Eumycota* also known as *true fungi* or *Eumycetes*. They also have a common ancestor that is a *monophyletic group*. The fungal group is also different from myxomycetes (slime molds) and oomycetes (water molds). Fungi are an important component of the soil micro biota dominating the soil biomass compared to bacteria depending on soil depth and nutrient conditions (Ainsworth & Bisby, 1995). Fungi are furthermore known to accumulate high amounts of metals (Morley et al., 1995). Due to this property, fungi has great importance to organisms growing in polluted habitats and has a potential for binding with heavy metals and removing waste waters and other aquatic substrata in natural environments (Gadd and White, 1989).

J. Bioresource Manage. (2014) 1(2): 38-51.

Pollution of soil by heavy metals affects the functioning of microorganisms and induces alteration in their population structure. Filamentous fungi were reported to exhibit considerable tolerance towards heavy metals and become dominant organisms in some polluted habitats (Martino et al., 2000). Fungi are known to tolerate and detoxify metals by several mechanisms including valence transformation, extra and intracellular precipitation and active uptake (Gadd, 1993).

During the last three decades like many other cities of Pakistan, Multan and Gujranwala has been going through a period of rapid industrial growth and large number of new industries has emerged in Multan and the Gujranwala area such as textile, leather goods and fertilizer. These Industries not only generate solid waste and liquid and cause contamination of soil and nearby water bodies by organic and inorganic waste of these industries (Chaudhary et al., 1999).

The aim of the present study is determine the tolerance index of micro fungal flora of contaminated soil and water samples against four different toxic metals (Cr,Cu,Cd and Pb). For this purposes soil and water samples were collected from area of Multan and Gujranwala. Physiochemical parameter of soil samples was analyzed to know the nature of soil and concentration of metals were also determined. The autoclaved distilled water was consumed to isolate the filamentous fungi from heavy metal contaminated soil and water samples. PDA was used as growth media to support the fungal proliferation.

MATERIALS AND METHODS

Sampling and Sampling Site

For present investigation Total 17 (Contaminated soil 10 and 07 waste water used for irrigation) samples were collected from Multan and Gujranwala peri-urban agricultural area of Pakistan. The water of both areas were contaminated by sewage and industrial effluents and contain heavy metals and toxic chemicals. Soil and water samples were collected for heavy metals analysis, filamentous fungal isolation and to check their tolerance index toward heavy metals.

Sterilization of Apparatus

J. Bioresource Manage. (2014) 1(2): 38-51.

Petri plates, media bottles, distilled water, McCartney bottles and syringes will be sterilized in autoclave. For sterilization purposes all apparatus will be autoclaved for 40 minutes at 121°C. After autoclaving all sterilized material will be dried in hot air oven at 95°C.

Media Preparation

Potato dextrose agar (PDA) was used as the growth media (Razak *et al*., 1999). PDA (29 g) was dissolved in 750 ml of sterilized water. After that, media was autoclaved at 121°C for 40 minutes. In order to retard the bacterial growth, 30mg/L streptomycin was also added in the media (Martin, 1950; Iram *et al*., 2012).

Preparation of Plates

After autoclaving the media, it was allowed to cool at room temperature. When temperature of media was at 60°C, 30 mg/lit of streptomycin was added in it to destroy the bacterial growth (Martin, 1950).

Then, this media was poured in petri plates and was left over for 24 hours so that the media could solidify. After solidification of media present in plates, these plates were placed at room temperature in an inverted position to avoid any water content (Martin, 1995). This work was all done in laminar flow to avoid any kind of bacterial growth.

Preservation and Identification of Fungi

Morphological studies were carried out by classical method in which the compound microscope was used at magnification of 100X, 400X and 1000X in which characterization was done on the basis of macroscopic (colonical morphology, shape, diameter, texture, appearance of colony) and microscopic (conidia shape, conidia structure, presence of reproductive structures, measurement on hyphal color, septation and presence of sterile mycelium) characteristics (Zafar *et al.,* 2006). Pure cultures of fungus were identified with the help of literature (Domsch et al.,1980; Barnett&Hunter1999). Fungi were preserved on slants of PDA for further studies.

Screening and Toxic Metal Experiment

For the screening of metal resistant fungal isolates, the PDA medium will be amended with 100ppm of (CU, Cd, Cr, Pd)

J. Bioresource Manage. (2014) 1(2): 38-51.

and a disk of mycelium will be inoculated aseptically on amended and unamended PDA plates in triplicates. The inoculated plates will be kept in an incubator at 28±1°C for 7 days. Effects of the heavy metals on the growth of the isolates will be estimated by measuring the colony diameter extension against the control (medium without metal). Metal Tolerance Index (T_i) will be calculated as the ratio of the total radius of the treated colony to that of the untreated colony (Ezzouhri, 2009).

$$T_i = \frac{D_t}{Du}$$

Where the D_t is the diameter (in cm) of treated colony and D_u is the diameter (in cm) of untreated colony (cm).

RESULTS AND DISCUSSION

In the present investigation, the resistance of fungi to heavy metals was studied by tolerance experiment. During April 2012 contaminated soils and water samples were collected from peri- urban agricultural areas of Multan and Gujranwala. A total of 17 fungal cultures were obtained for present study for detailed investigation of heavy metal tolerance. The main purpose of the present study was to test the tolerance index of filamentous fungi of genra i.e., *Aspergillus* , *Pencillum* and *Fusarium* against heavy metals Cr , Pb, Cu and Cd.

Heavy Toxic Metal Content In Contaminated Soil And Water

Growth of fungal isolates at Copper

The relative toxicity of various metals on growth rate can be expressed by assessing the tolerance index. Tolerance index was assessed to evaluate the effect of heavy metal on the growth rate of the fungi. This tolerance index was calculated by measuring growth in the presence of metal divided by the growth of the fungi in the same period in the absence of metal. The present (Figure 1) regarding Cu metal content in contaminated soil and water samples collected from Multan and Gujranwala showed that *Aspergillus Versicolor* show maximum tolerance index 2.88 at 100 ppm of Cu concentration in Gujranwala water and there was 2 time more growth as compare to control. While *Aspergillus flavus* show minimum tolerance

J. Bioresource Manage. (2014) 1(2): 38-51.

index, i.e 0.5 at 100 ppm of Cu concentration in Multan water and it shows less growth as compare to control.

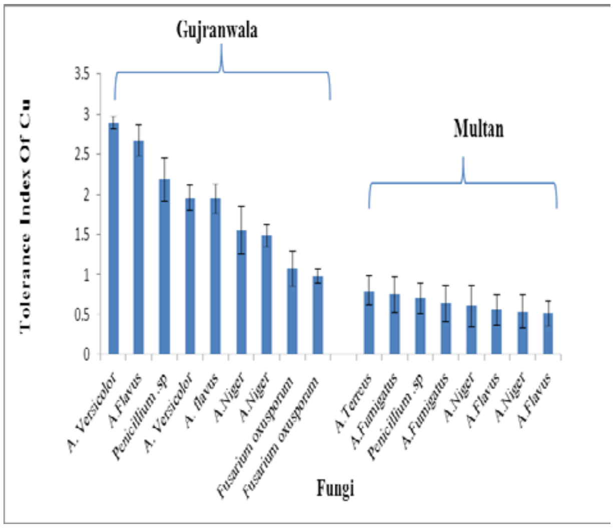

Figure 1: Tolerance Index of fungi isolated from Multan and Gujranwala soil and water samples against Cu.

Growth of fungal isolates at Chromium

The present study regarding Cr metal content in contaminated soil and water samples collected from Multan and Gujranwala shows that *Penicillium sp* show maximum tolerance index 2.1 at 100ppm of Cr concentration in Gujranwala soil and it shows 1 time more growth as compare to control. *Aspergillus niger* show minimum tolerance index 0.5 at 100ppm of Cr concentration in Multan water and it shows less growth as compare to control. (Figure 2).

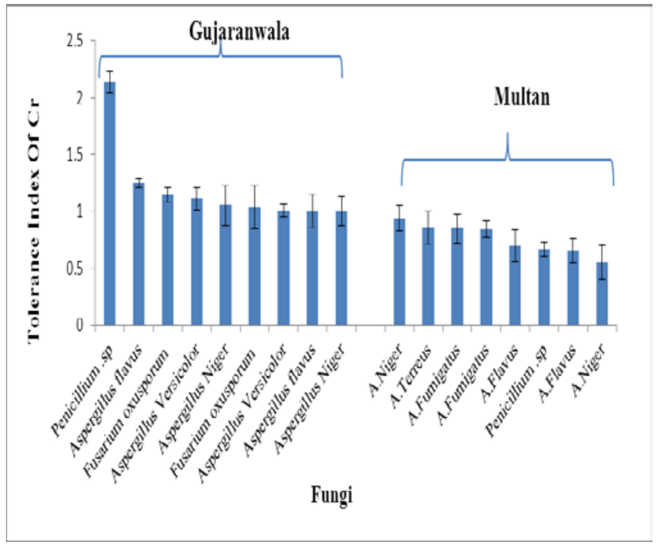

Figure 2: Tolerance Index of fungi isolated from Multan and Gujranwala soil and water samples against Cr.

Growth of fungal isolates at Cadmium

The present study as regards Cd metal content in contaminated soil and water samples collected from Multan shows that *A. Flavus* show maximum tolerance index 4.8 at 100ppm of Cd concentration in soil of Gujranwala and it shows 3 times more growth as compared to control. *Aspergillus niger* show minimum tolerance index 0.2 at 100ppm of Cd concentration in water and it shows less growth as compare to control

J. Bioresource Manage. (2014) 1(2): 38-51.

(Figure 3). Mean growth decline shows significant change on growth behavior.

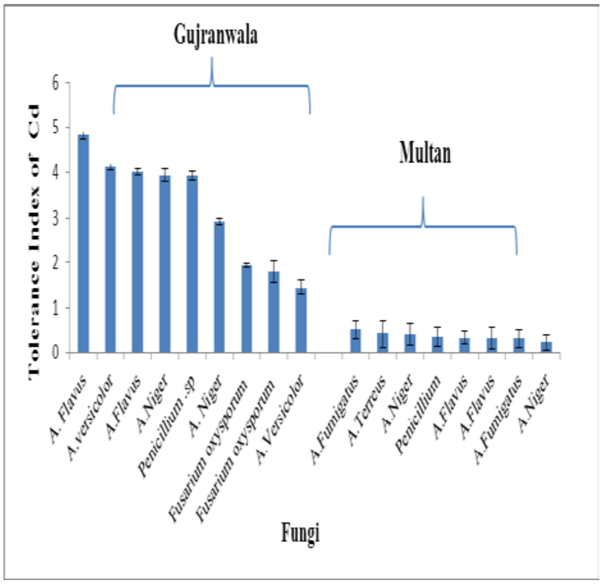

Figure 3: Tolerance Index of fungi isolated from Multan and Gujranwala soil and water samples against Cd.

Growth of fungal isolates at Lead

Figure 4 shows Pb metal content in contaminated soil and water samples collected from Multan and Gujranwala shows that *Aspergillus versicolor* exhibit maximum tolerance index 2.6 at 100ppm of Pb concentration in water of Gujranwala, as well as 2 time more growth as compared to control. *Aspergillus niger* show minimum tolerance index 0.5 at 100 ppm of Cu concentration in water and it less growth as compare to control.

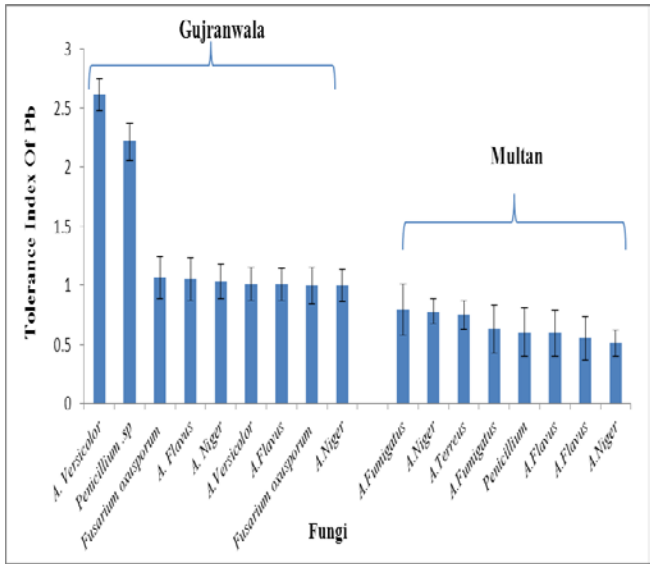

Figure 4: Tolerance Index of fungi isolated from Multan and Gujranwala soil and water samples against Pb.

It is well know that a long term exposure of heavy metal in water and sediment can produce a significant modification of their microbial populations, reducing their activity and their number (Doelman et al., 1994).The high content of heavy metal in treated soil is likely due to long term application of waste water containing these heavy metals (Malik & Jaiswal, 2000).

J. Bioresource Manage. (2014) 1(2): 38-51.

Describing the ability to grow at high metal concentrations (Wainwright & Gadd, 1997) distinguishes fungi that is tolerant of and resistant to heavy metals. Resistance involves detoxification mechanisms produced in direct response to the toxic metal, whereas tolerance is defined as the ability to cope with toxicity by means of intrinsic properties of the organism including impermeable, usually pigmented, cell walls, as well as excretion of various extracellular high-molecular mass substances (Nies, 1999).

Determination of tolerance index toward the heavy metal indicated tolerance capability of individual isolate. Some were sensitive, moderately tolerant and tolerant. This tolerance index was taken as the measured growth in the presence of metal divided by the fungi in the same period in the absence of fungi.

The tolerance index of species of *Aspergillus flavus*, *Aspergillus versicolor*, *Fusarium* oxysporum , *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus fumigatus* and *Penicillium sp* were observed in the particular conditions .

The results of present study *Aspergillus* was the dominant genera among the Multan and Gujranwala soils and water, and exhibit the highest tolerance index toward soil and water. In Pakistan, heavy metals contamination is an important environmental and economic issue, The combination of poorly-planned effluent disposal techniques and a rapidly growing population have led to a gradual accumulation of heavy metals in soil and water of Pakistan. In Pakistan the EPA has reported the heavy metals including lead, arsenic, chromium, mercury and zinc etc. level in effluents and soil near tanneries and textile mill greatly exceed the safety limit levels standardized by the National Environment and Quality Standard (NEQS) (Khan, 2001).

The present work evaluated the difference in metal resistance among different isolates of fungus from contaminated soil and water of Multan and Gujranwala. Similar study reported by (Iram *et al*., 2012) highest tolerance of Cr in soil because heavy metals enter the soil and get fixed to the soil components that tend to accumulate large quantities of heavy metals in soil which persist and have long lasting effects in the soil.

According to previous reports (Rothbaum *et al*., 1986), higher concentration of Cd is because of super phosphate fertilizers.

According to (CCME, 1992) the concentration of Pb and Cu in the water samples was found to be above the permissible limits respectively. It is due to long time exposure of water and sediment to heavy metals, which can produce considerable modification of their microbial populations, reducing their activity and their number. (Doelman et al.,1994).

Copper metal at high concentration is toxic to *A. terreus* and *A. alternata* as their growth markedly decreased with increasing copper concentrations in the growth medium.

Similar results were also observed where fungi isolated from metal contaminated agricultural soil belonged to genera *Aspergillus, Penicillium, Alternaria, Geotrichum, Fusarium* and *Trichoderma* showed a significant tolerance to heavy metals. The minimum inhibitory concentration (MIC) ranged from 0.6 to 9 mg/ml for Cu depending on the isolate (Zafar *et al.*, 2007)

According to the research of Baharani *et al.,* (2003), *Aspergillus niger* was found to accumulate more lead in the mycellial mat as the concentration increased. When compared to the age of cells on 7th day, mycellial mats were found to accumulate more lead. *Aspergillus niger* was seen to grow even at 100mg/l concentration without any inhabitation.

The high metal tolerant species most probably have developed the physiological adaptation mechanism for surviving in elevated Cd concentrations (Balamarugan, Schaffner 2006, Gonzalez-Chavez *et al.* 2002). In accordance with these findings, it was reported that the genera *Aspergillus* are of high capacity to biosorb cadmium and other heavy metals (Volesky 1990; Zafar *et al.* 2007; Lopez-Errasquin and Vasques 2003). Similar metal tolerance differences among the isolates of the same genus have also been observed in this study.

J. Bioresource Manage. (2014) 1(2): 38-51.

In the present investigation, no general pattern was found which could fit all the strains. Every strains of same species can have a different physiological adaptation to react differently even with the same metal at the same concentration in the water and soil of Multan and Gujranwala.

CONCLUSION

It is concluded from our research that *Aspergillus* is the main dominant and wide occurring genera in heavy metal contaminated samples which indicates its resistance towards harmful heavy metals and the ability to show highest tolerance index toward copper, cadmium and lead. The tolerance and the resistance of the isolates depended much more on the fungus tested than on the sites of its collection. This variation may be explained by the development of tolerance or adaptation of the fungi to heavy metals. From all the collected samples the Gujranwala soil and water show more tolerance toward the heavy metals as compared to Multan area.

This study recommends that the species of *Aspergillus* and *Penicillium* found in the soil and water samples of Gujranwala and Multan should be utilized for the bioremediation process. Fungi have been widely used in bioremediation of industrially polluted soils and waters, specifically in the removal of hydrocarbons and heavy metals (Akhtar, Mohan, 1995; Khan, 2001; Potin et al., 2004). The results obtained confirmed that the response of isolates to heavy metals depended on the metal tested, its concentration in the medium and on the isolate under consideration.

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